Fermentative characteristics of *Leuconostoc mesenteroides* ATCC 8293(T) in Palm (*Borassus flabellifer*) sap at different temperatures.

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Abstract— Inflorescence of palm tree (*Borassus flabelliffer*) is chopped to produce nutritionally rich palm sap early in the morning in several parts of India. Healthy palm sap drink becomes alcoholic beverage with sour to bitter taste due to the action of microbes with time and temperature. Hence a fermentative characteristic of one of such microbe, *Leuconostoc mesenteroides* ATCC 8293(T) in fresh palm sap that was sterilized at 60°C for 25 min was done so as to intervene the fermentation of palm sap by *Leuconostoc mesenteroides* ATCC 8293(T). Insignificant fermentation was observed beyond 60°C for 15 min in nutritionally rich palm sap

Index Terms— Palmyra Palm, sterilization, *Leuconostoc mesenteroides*, *Borassus flabellifer*, temperature, time, Lactic acid bacteria.

1 INTRODUCTION

Fresh Palmyra palm (*Borassus flabellifer*) sap tapped early in the morning is sweet and clear. This nutritionally rich sweet and clear drink becomes sour or bitter as the sun rises due to oxido-reductive fermentative process [1]. In this uncontrolled spontaneous fermentative process microbes present on the contact surface area produces lactic acid, ethanol and acetic acid [2].Decrease in the sugar content in palm sap during initial stage of fermentation is due to the microbial metabolic activity[3].Change in the colour of the fresh palm sap from transparent to whitish colour is due to the production of a gum probably dextrans by lactic acid bacteria, and in addition to this heavy suspension of microbes gives milky-white colour to the palm sap[4]. Reduction in pH during the initial fermentation is due the production of lactic acid by Lactic acid producing bacteria[5]. Volatile profile of the palm sap is due to the ethanol and acetic acid produced by the microbes[6]. Temperature of the environment and time of exposure are the important parameter that influences the fermentation as it controls the viability of the cells, growth rate of the isolate, exponential phase of the strain, enzyme activity of the microbes, and function of the semipermeable membrane of the cells[7]. *Leuconostoc mesenteroides* is a mesophilic bacteria and is responsible for sour taste to the medium due to organic acid production and reduction of pH[8][9]. Hence, optimizing the growth parameters of *Leuconostoc mesenteroides* is very important so as to intervene the fermentation to produce health drink.

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2 MATERIALS AND METHODS

2.1 Sample Collection

Freshly tapped palm sap (*Borassus flabellifer* L.) was collected from Sajipa of Dakshina Kannada District (Karnataka, India) at around 6.00 AM over 14 tapping process in the month of December. Environmental temperature varied from 18 to 24°C. 50 mL of the samples are collected directly from earthen pot collection into a sterile 50 mL sample collection vessels under sanitary condition and transported to the laboratory of the Department of Biotechnology, P. A. College of Engineering, Mangalore, in an insulated container maintained at 4°C within 30 min. After filtration using sterile muslin filter cloth samples was stored at 4°C until analysis and if any delay then preserved at –50°C in a deep freezer (Model C340, New Brunswick Scientific, England).

2.2 Chemicals

Chemicals are manufactured and supplied by Merck Limited (Mumbai, India), and reagents were prepared as per the current American Chemical Society specifications [10]. Nutrient agar, deMan, Rogosa and Sharpe Agar (MRSA), deMan, Rogosa and Sharpe broth (MRSB) were procured from Himedia, Mumbai and prepared as per manufacturer's instructions. Ingradienta and media were sterilized in moist heat at 15 lbs pressure for 15 min. Inoculated plates were incubated at 37°C in incubater (REMI, Cochin) and the colonies are counted using digital colony counter (Systronics, Mumbai).

2.3 Sterilization of the palm sap

Sterelization of the palm sap was carried out at 60°C for 25 min. Sterilization was carried out in water bath (Rotek Instruments, Kerala) in 250 mL capacity flask. Properly plugged and wrapped flasks were arranged in the water bath with sufficient space to facilitate the moist heat penetration. Palm sap exposed to various temperatures for different intervals of time was then analyzed for total protein content, total lipid content, Vitamin C, reducing sugar, non-reducing sugar, glucose, su-

crose, ethanol, mould count and bacterial count to optimize the parameter.

2.4 Microorganisms and cultural preparations

Leuconostoc mesenteroides ATCC 8293(T) was grown in MRSB. Ammonium solution (1:1) or 2N HCl solution was used to adjust the pH of MRSB to and was sterilized at 121°C in moist heat at 15 lbs pressure for 15 min. After cooling, 250 mL of MRSB was inoculated at *Leuconostoc mesenteroides* ATCC 8293(T) and incubated at a temperature of 30°C for 3 days under semi-aerobic condition, when the concentration of cells in optical density of the medium was reached 1.3 nm. Samples were drawn for analysis in quadruplicates after appropriate intervals of time. After attaining appropriate growth, LAB cells were recovered from the MRSB by centrifugation at relative centrifugal force (RCF) of 3,500xg for 10 min(C-24BL/CRP24 model centrifuge, Remi Laboratory Instruments, Mumbai, India). Pellets so obtained were separated and stored at stored at 40°C in a cold store for further stud [11].

2.5 Kinetics for palm sap fermentation

Fermentative characteristics of the lactic acid bacteria and effect of time and temperature on fermentaion were studied in the 1L laboratory fermenter under [12]. Growth of *Leuconostoc mesenteroides* ATCC 8293(T) in sterile palm sap of pH 6.5 with associated changes in the components of the medium were carried at temperature of 10, 20, 30, 40, 50, and 60°C for time interval of 5 h up to 50 h was carried out. Palm sap so treated was then analyzed for total protein content, lipid content, Vitamin C, reducing sugar, glucose, sucrose, ethanol, and lactic acid bacterial count on MRSB by drawing samples at different intervals of time to optimize the growth parameter.

2.6 Proximate analysis during incubation

MRSB was clarified at RCF of 1681.1x*g* for 5 min at 4°C and clear samples were used for proximate analysis. Portable Glass electrode pH meter (Systronics, Mumbai) was used to measure pH. Inoculated MRSB or MRSA was incubated at 30°C in an incubator (Rotek Instruments, Kerala). Infra-red thermometer (Quicktemp 826 T4, Austria) was used to measure temperature of the samples. Visible spectrophotometer (Systronics, Mumbai) was used to measure absorbance of the cell free samples at 10, 20, 30, 40, 50 and 60°C in 1 cm Quartz cell at 420 nm. Total protein content in the samples at 10, 20, 30, 40, 50, or 60°C were estimated by Lowry's method and values were expressed in mg/Ml [13]. Total lipid content in palm sap at 10, 20, 30, 40, 50, or 60°C were estimated after extraction using chloroform-methanol extraction method followed by reaction with sulfuric acid and vanillin phosphoric acid reagent and values were expressed in percentage (w/v) [14]. Vitamin C content in the samples at 10, 20, 30, 40, 50, or 60°C were estimated by Redox Titration methods using 2, 4-dinitrophenyl hydrazine (DNPH) dye and standard ascorbic acid, and values were expressed as mg/mL [15]. Reducing sugar in palm sap at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 h were estimated dinitrosalicylic acid reagent, and values were expressed in percentage (v/v) [16]. Glucose and Sucrose 10, 20, 30, 40, 50, or 60°C were estimated using High sensitive Glucose and Sucrose Assay kit provided by EMerck, India, and values were expressed in percentage (w/v).

Changes in ethanol content in the palm sap was estimated at 10, 20, 30, 40, 50, or based on the colorimetric reaction of ethanol with sodium dichromate, and values were expressed in percentage (v/v) [17]. Changes in mould count and bacterial count of the palm sap at 60, 70, 80, 90, 100, 110 or 120°C were performed as per APHA method and values were expressed in cfu/mL [18]. The samples were collected and analysed in quadruplicate.

2.7 Statistical analysis

MRSB were collected at 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 h of incunation and analysed in quadruplicate by One way-analysis of variance (ANOVA) using the Fisher's least significant difference (LSD) test to estimate the significant differences between each sample ($p \leq 0.05$) using Statgraphics Centurion XV software (Statpoint Technologies Inc., Warrenton, VA, USA).

3 RESULTS

3.1 Effect of Time and Temperature on the absorbance of palm sap

Changes in optical density of the palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50, or 60°C (LAB-AB-10, LAB-AB-20, LAB-AB-30, LAB-AB-40, LAB-AB-50, and LAB-AB-60, respectively) for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 1.

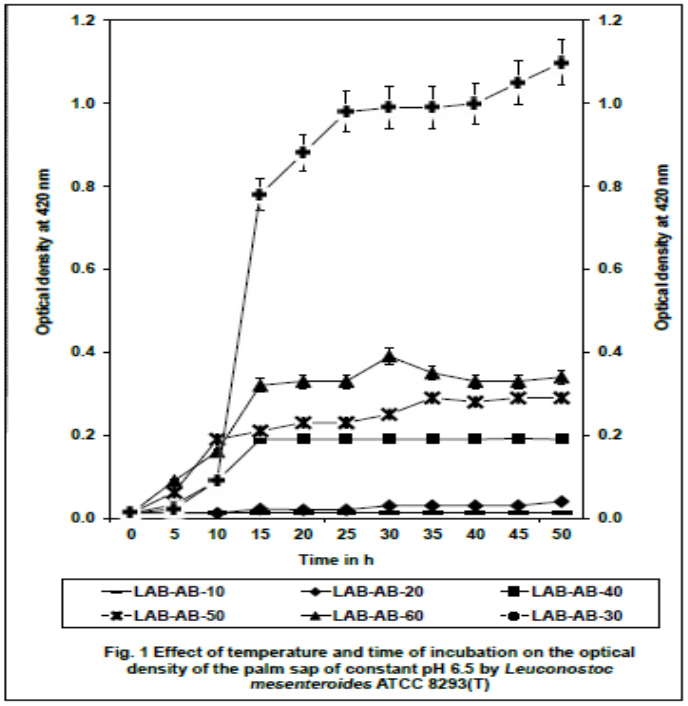

Fig. 1 Effect of temperature and time of incubation on the optical density of the palm sap of constant pH 6.5 by *Leuconostoc mesenteroides* ATCC 8293(T)

Freshly tapped palm sap is transparent, less viscous and without any colour, but due to microbial activity it translates to opaque, more viscous and whitish in appearance. To study the effect of temperature and time on palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) and associated changes in optical density of the palm sap, different batched of freshly tapped palm sap samples were inoculated with 4.00×10^5 cfu/mL of the isolate and incubated at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 h. Optical density of these samples were compared with its initial samples and with the samples incubated at 10°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 60 h. Here, at 30°C optical density of the palm sap increased slowly till 10 h of incubation and there after drastic increase in the optical density of the palm sap was noticed up to 15 h of incubation rate of increase of optical density was reduced in comparison to the rate of increase in between 10 and 15 h of incubation.

3.2 Effect of Time and Temperature on ethanol production

Effect of temperature of incubation on the ethanol content of the palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) in palm sap at 10, 20, 30, 40, 50, or 60°C 60°C (LAB-EOH-10, LAB-EOH -20, LAB-EOH-30, LAB-EOH-40, LAB-EOH-50, and LAB-EOH-60, respectively) for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 2.

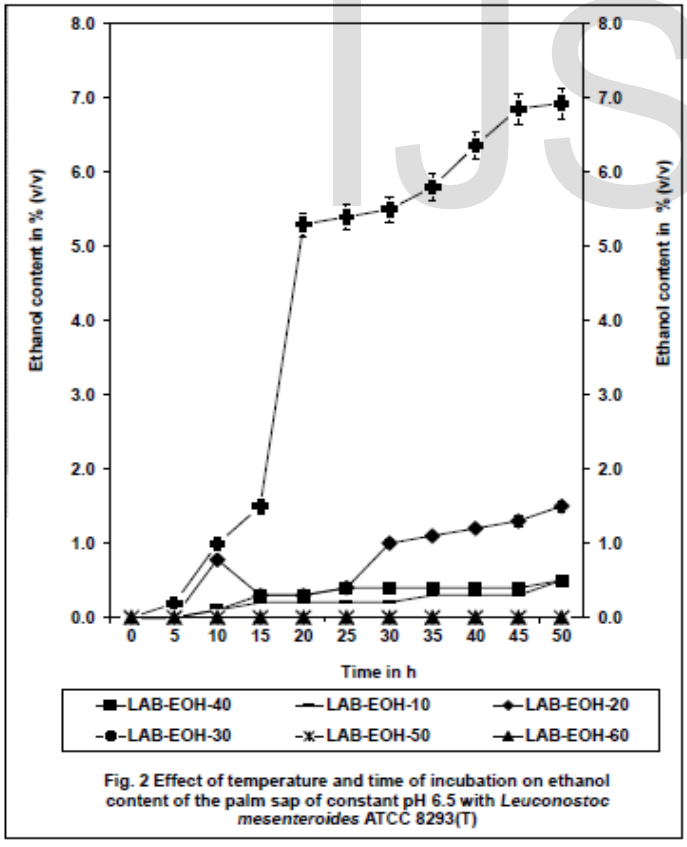

Fig. 2 Effect of temperature and time of incubation on ethanol content of the palm sap of constant pH 6.5 with *Leuconostoc mesenteroides* ATCC 8293(T)

Figure 1 and 2 illustrates the interaction between the two variables of pure culture of lactic acid bacteria such as temperature and time but at constant pH of 6.5 on the absorbance and ethanol production, respectively. Changes in the physico-chemical properties of palm sap incubated with pure cultures of *Leuconostoc mesenteroides* ATCC 8293(T) was carried out at controlled physical factors such as temperature and time of incubation and pH of the palm sap that are important for the fermentation of ethanol. Freshly tapped palm sap collected from inflorescence of *Borrasus flabellifer* was transparent without any colour and less viscous with an optical density of 0.01 at 420 nm, during 50 h of incubation at 30°C optical density of the palm sap increased to 1.10 at 420 nm. During this period ethanol accumulated in the palm sap was 8.86% (v/v), where as the alcohol accumulated during this period at 20°C is only 6.44% (v/v). We were not able to establish a significant (p>0.05) change in the amount of alcohol production at other temepraturs for varied periods.

3.3 Effect of Time and Temperature on viable count of plam sap

Effect of temperature of incubation on the count of *Leuconostoc mesenteroides* ATCC 8293(T) in palm sap at 10, 20, 30, 40, 50, or 60°C (LAB-COUNT-10, LAB- COUNT -20, LAB-COUNT-30, LAB-COUNT-40, LAB COUNT-50, and LAB-COUNT -60, respectively) for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 3.

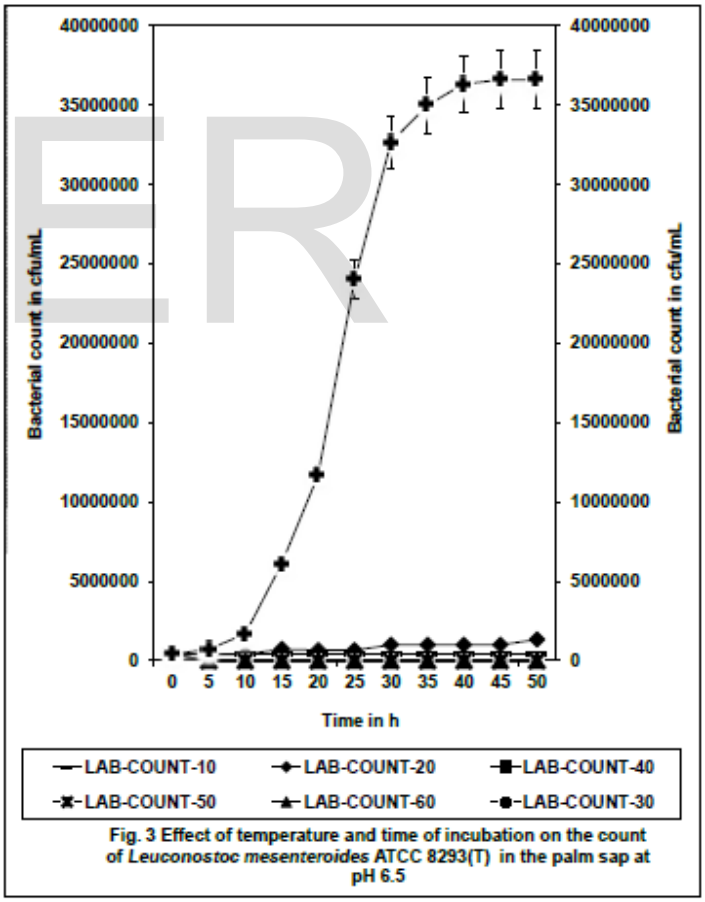

Fig. 3 Effect of temperature and time of incubation on the count of *Leuconostoc mesenteroides* ATCC 8293(T) in the palm sap at pH 6.5

Viable Count in palm sap that was sterilized and inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) was increased from 4.00×10^5 cfu/mL to 3.66×10^7 cfu/mL during 50 h of incubation at 30°C. The palm sap incubated at 10, 40, 50 and 60°C for 50 h did not show any significant (p>0.05) change in viable count in comparison to the viable count of its initial samples, as confirmed by One way ANOVA with *post hoc*

Tukey's test. However, viable count of the palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) and incubated for 20, and 30°C showed 3 and 92 folds increase in the viable count. Overall significant effect of temperature of incubation on the increase in viable count of LAB of the palm sap in these samples remained at 5% level of significance, as indicated by One way ANOVA with *post hoc* Tukey's test.

3.4 Effect of Time and Temperature on sucrose utilization by LAB in palm sap

Effect of temperature of incubation on the sucrose level of palm sap inoculated with of *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50, or 60°C (LAB-SU-10, LAB-SU -20, LAB-SU-30, LAB-SU-40, LAB-SU-50, and LAB-SU-60, respectively)for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 4.

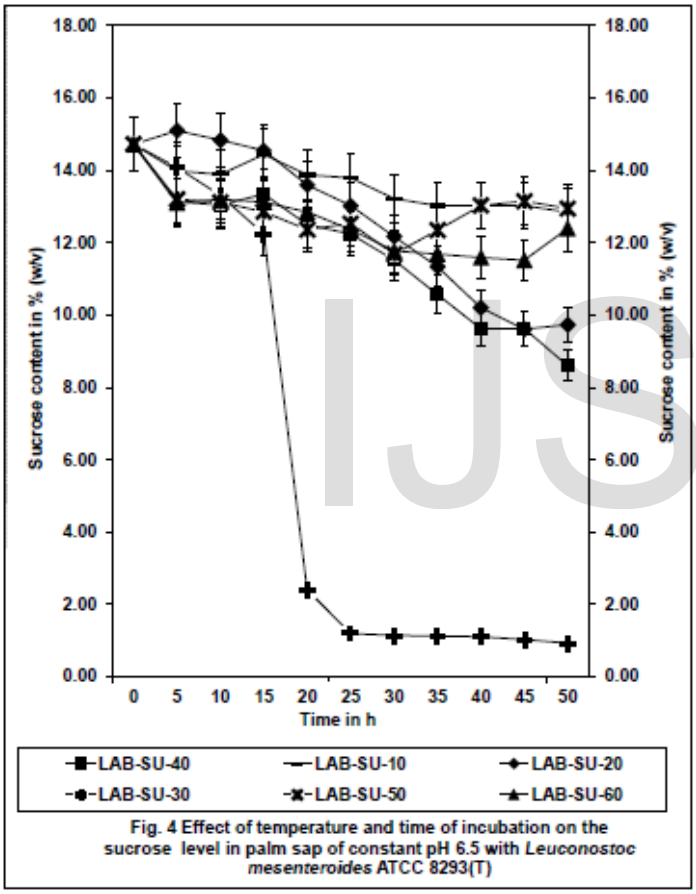

Fig. 4 Effect of temperature and time of incubation on the sucrose level in palm sap of constant pH 6.5 with *Leuconostoc mesenteroides* ATCC 8293(T)

Utilisation of sucrose in palm sap that was sterilized and inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) was investigated. Sucrose level in the sample during 50 h of incubation at 30°C was reduced from 14.72 ±0.09% 0.93 ±0.04%. One way ANOVA with *post hoc* Tukey's test was able to establish a significant (p<0.05) level of sucrose in samples incubated at 20, 30, and 40°C for 50 h in comparison to the sucrose of its initial samples. Sucrose in the palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) and incubated for 20, 30, and 40°C showed 2, 16 and 2 folds decrease in the sucrose level. Overall significant effect of temperature of incubation on the decrease in sucrose by LAB of the palm sap in these samples remained at 5% level of significance, as indicated by One way ANOVA with *post hoc* Tukey's test.

3.5 Effect of Time and Temperature on glucose utilization by LAB in palm sap

Effect of temperature of incubation on the glucose level of palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50, or 60°C (LAB-GL-10, LAB-GL -20, LAB-GL-30, LAB-GL-40, LAB-GL-50, and LAB-GL-60, respectively)for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 5.

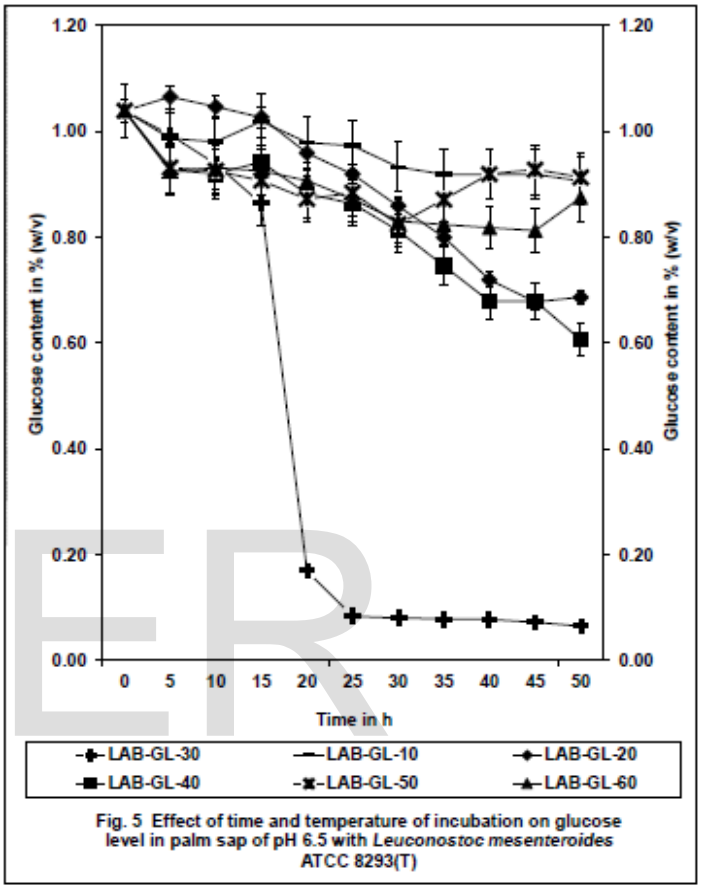

Fig. 5 Effect of time and temperature of incubation on glucose level in palm sap of pH 6.5 with *Leuconostoc mesenteroides* ATCC 8293(T)

During 50 h of incubation at 30°C, glucose level in the palm sap was reduced from 1.04 ±0.002% 0.07 ±0.001%. One way ANOVA with *post hoc* Tukey's test was not able to establish a significant (p>0.05) level of difference in glucose levels in between samples incubated at 10, 50, and 60°C for 50 h in and the glucose levels in the initial samples. Glucose levels in the samples inoculated and incubated with *Leuconostoc mesenteroides* ATCC 8293(T) for 20, 30, and 40°C showed 1.5, 16 and 1.7 folds reduction in the glucose level. Overall significant effect of temperature of incubation on the decrease in glucose of LAB of the palm sap in these samples remained at 5% level of significance.

3.6 Effect of Time and Temperature on protein content in palm sap

Effect of temperature of incubation on the protein content of palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50, or 60°C(LAB-TPC-10, LAB-TPC -20, LAB-TPC-30, LAB-TPC-40, LAB-TPC-50, and LAB-TPC-60, respectively) for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 6

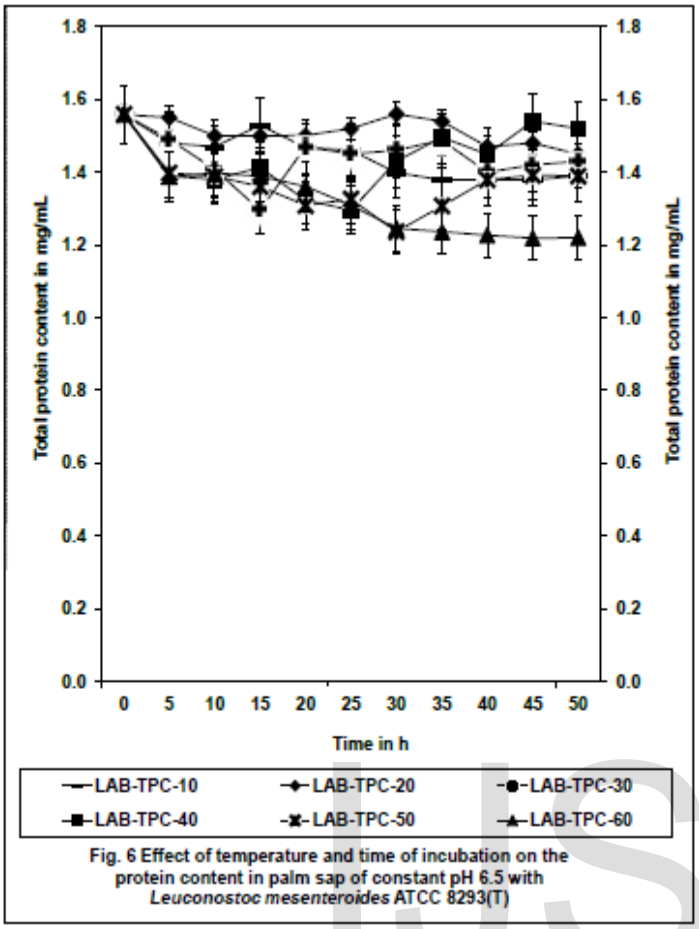

Fig. 6 Effect of temperature and time of incubation on the protein content in palm sap of constant pH 6.5 with *Leuconostoc mesenteroides* ATCC 8293(T)

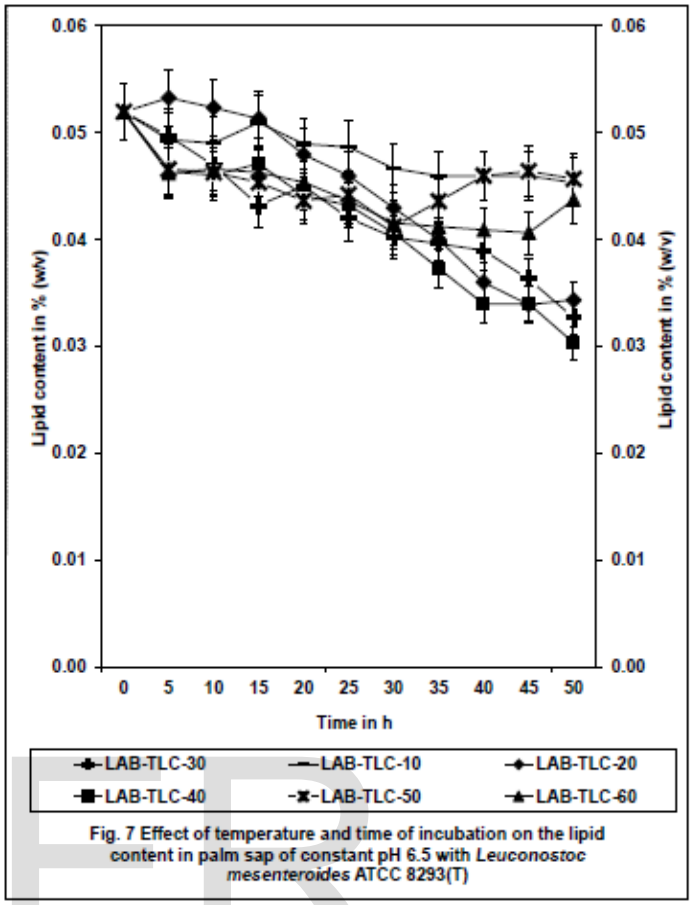

Fig. 7 Effect of temperature and time of incubation on the lipid content in palm sap of constant pH 6.5 with *Leuconostoc mesenteroides* ATCC 8293(T)

Protein content in the initial palm sap was 1.56±0.53 mg/mL, and the protein content during 50h of incubation of incubation of *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50 and 60°C was increased, respectively, to 1.39±0.73, 1.45±0.93, 1.43±0.78, 1.52±0.89, 1.39±0.89, and 1.88±0.53 mg/mL. One way ANOVA with *post hoc* Tukey's test was not able to establish a significant (p>0.05) difference in protein content in between samples incubated at 10, 20, 30, 40, 50 and 60°C for 50 h, and in comparison to protein content of the initial samples.

3.7 Effect of Time and Temperature on lipid content in palm sap

Effect of temperature of incubation on the lipid content of palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50, or 60°C (LAB-TLC-10, LAB-TLC -20, LAB-TLC-30, LAB-TLC-40, LAB-TLC-50, and LAB-TLC-60, respectively) for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 7. Lipid content in the initial palm sap was 0.05±0.005 mg/mL, and the Lipid content during 50h of incubation of incubation of *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50 and 60°C was increased, respectively, to 0.05±0.007, 0.03 ±0.006, 0.03 ±0.009, 0.03 ±0.008, 0.03±0.008, and 0.04±0.007 mg/mL. One way ANOVA with *post hoc* Tukey's test was not able to establish a significant (p>0.05) difference in Lipid content in between samples incubated at 10, 20, 30, 40, 50 and 60°C for 50 h, and in comparison to protein content of the initial samples.

3.8 Effect of Time and Temperature on Vitamin C content in palm sap

Effect of temperature of incubation on the Vitamin C of palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50, or 60°C(LAB-VC-10, LAB-VC -20, LAB-VC-30, LAB-VC-40, LAB-VC-50, and LAB-VC-60, respectively) for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h is illustrated in the figure 8. Vitamin C in the initial palm sap was 0.062±0.008 mg/mL, and the Vitamin C during 50h of incubation of incubation of *Leuconostoc mesenteroides* ATCC 8293(T) at 10, 20, 30, 40, 50 and 60°C was increased, respectively, to 0.054±0.006, 0.041 ±0.008, 0.039 ±0.007, 0.036 ±0.005, 0.055±0.006, and 0.052±0.007 mg/mL. One way ANOVA with *post hoc* Tukey's test was not able to establish a significant (p>0.05) difference in Vitamin C in between samples incubated at 10, 20, 30, 40, 50 and 60°C for 50 h, and in comparison to protein content of the initial samples. Effect of temperature on *Leuconostoc mesenteroides* ATCC 8293(T) in palm sap at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 h was studied. Here, interaction between the two variables of incubation of lactic acid bacteria isolates such as temperature and time but at constant pH of 6.5 on the absorbance and the ethanol content was analysed. However, One way ANOVA with *post hoc* Tukey's test was not able to establish as significant (p>0.05) difference in samples collected above 40 °C and below 20°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 or 50. Here interesting note here that One way ANOVA with *post hoc* Tukey's test was not able to establish as significant (p>0.05)

difference protein content, lipid content and Vitamin C content in samples incubated at 10, 20, 30, 40, 50 or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 h.

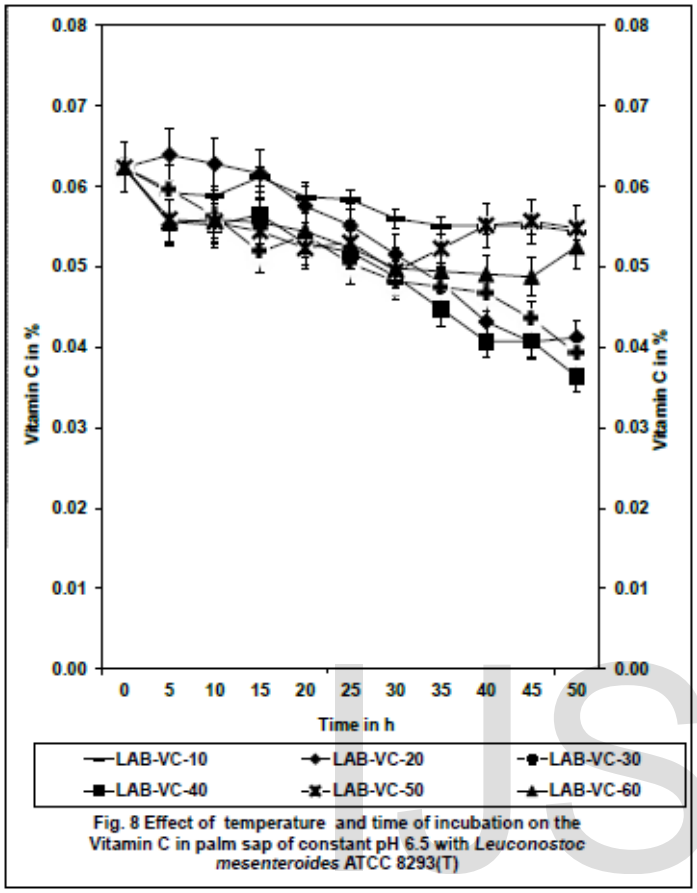

Fig. 8 Effect of temperature and time of incubation on the Vitamin C in palm sap of constant pH 6.5 with *Leuconostoc mesenteroides* ATCC 8293(T)

4 DISCUSSION

Set of experimental variables such as temperature of incubation and time of exposure to these two variables are very important factors of fermentation. Standardizing these variables helps in fixing the unfavourable conditions so as to intervene the fermentation of the palm sap to prevent utilisation of the nutrients by microbes. Here maximum to minimum ranges of temperature was interacted with maximum to minimum ranges of time at constant pH. Since the Lactic acid bacteria, *Leuconostoc mesenteroids* subspecies *mesenteroids* ATCC 8293(T) was dominating species isolated from palm sap of the seven palm trees of Sajipa Village we have selected this species to perform the kinetics of palm sap fermentation. Characteristics of the predominating microbial isolates of palm sap, *Leuconostoc mesenteroids* subspecies *mesenteroids* ATCC 8293(T) at different temperature and time was performed to determine the favourable and unfavourable condition of fermentation so as to intervene it to produce health drink, or otherwise it is been used as an alcoholic drink.

To study the effect of temperature and time on palm sap inoculated with *Leuconostoc mesenteroides* ATCC 8293(T) and associated changes in the palm sap, different batched of freshly tapped palm sap samples were inoculated with 4.00×10^5 cfu/mL of the isolate and incubated at 10, 20, 30, 40, 50, or 60°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h at pH 6.5. Optimum temperature of the *Leuconostoc mesenteroides* ATCC 8293(T) in palm sap was 30°C as the maximum absorbance, ethanol production, carbohydrate utilisation and maximum growth was exhibited in comparison to the other temperatures such as temperature 10, 20, 40, 50, or 60°C all along 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 h of incubation at pH 6.5. Optimum growth rate of *Leuconostoc mesenteroides* is 30°C at pH 6-6.5. However, optimum temperature for bacteriocin production is 25°C at 5.5 pH and this bacteria is responsible for sour taste due to organic acid production and reduction of pH [9]. Temperature is an important external factor that effects the fermentation in batch mode. However, there are report sighting that temperature tolerance are exhibited by in yeast during fermentation [19].

Our present study shows a maximum rate of ethanol production during 16 to 20 h of incubation and over all ethanol production was very high during the initial 24 hours of fermentation at 30°C and pH 6.5. Previous work suggest that ethanol accumulation during the initial 24 h of incubation is very high and microbes grows rapidly at temperature between 25°C and 33°C, and optimum alcohol production is at 30°C and 37°C[20]. Temperature of fermentation medium plays an important role in the production of ethanol, viability of cells, growth rate of the microbes, enzyme activity of the alcohol producer, exponential phase of the ethanol producing strains and properties of semipermiable membrane [21].

5 CONCLUSION

Freshly tapped palm sap is sweet and clear, but microbial activity changes it to milky white and sour. *Leuconostoc mesenteroides* ATCC 8293(T) grown optimally at 60°C for 15 min, beyond which least degradative changes takes place in nutritionally rich palm sap. Degradative activity of lactic acid bacteria can be intervened by adjusting the temperature of the medium and period of incubation to a level beyond the optimum levels where least degradtive changes takes place. Present study gives valuable information for the intervention of the palm sap from fermentation.

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